

Phylogenetic Analysis of the Tetrasporalean Genus *Asterococcus* (Chlorophyceae) Based on 18S Ribosomal RNA Gene Sequences

Atsushi NAKAZAWA and Hisayoshi NOZAKI

Department of Biological Sciences, Graduate School of Science, University of Tokyo,
Hongo 7-3-1, Bunkyo-ku, Tokyo, 113-0033 JAPAN

(Received on October 30, 2003)

Nucleotide sequences (1642 bp) from 18S ribosomal RNA genes were analyzed for 100 strains of the clockwise (CW) group of Chlorophyceae to deduce the phylogenetic position of the immotile colonial genus *Asterococcus* Scherffel, which is classified in the Palmellopsidaceae of Tetrasporales. We found that the genus *Asterococcus* and two unicellular, volvocalean genera, *Lobochlamys* Pröschold & al. and *Oogamochlamys* Pröschold & al., formed a robust monophyletic group, which was separated from two tetrasporalean clades, one composed of *Tetraspora* Link and *Paulschulzia* Skuja and the other containing the other palmellopsidacean genus *Chlamydocapsa* Fott. Therefore, the Tetrasporales in the CW group is clearly polyphyletic and taxonomic revision of the order and the Palmellopsidaceae is needed.

Key words: 18S rRNA gene, *Asterococcus*, Palmellopsidaceae, phylogeny, Tetrasporales.

Asterococcus Scherffel (1908) is a colonial green algal genus that is characterized by an asteroid chloroplast in the cell and swollen gelatinous layers surrounding the immotile colony (e. g., Ettl and Gärtner 1988). This genus is generally assigned to the Tetrasporales (e. g., Lemmermann 1915, Tiffany 1934, Smith 1950, Ettl 1964, Fott 1971, Ettl and Gärtner 1988). However, the systematic position of *Asterococcus* at the family level varies according to the author. Lemmermann (1915) classified *Asterococcus* in the Palmellaceae. Korshikov (1953) considered this genus a member of the dendroid family Chlorangiellaceae because his alga produced dendroid, gelatinous colonies. Fott (1971) distinguished *Asterococcus* from other members of the Tetrasporales and established a new family, the Asterococcaceae.

Recently, Ettl and Gärtner (1988) included *Asterococcus* in the family Palmellopsidaceae, because cells of this genus have contractile vacuoles and lack pseudoflagella in the non-attached vegetative colony.

Patricia et al. (2000) revealed that *Asterococcus* belongs to the clockwise (CW) group of Chlorophyceae, based on the CW orientation of the flagellar apparatus in *A. superbus* (Cienkowski) Scherffel. Nakazawa et al. (2004) carried out a taxonomic study of *Asterococcus* based on the comparative morphology of many strains and molecular phylogenetic analyses of the Rubisco large subunit (*rbcL*) gene sequences. However, the phylogenetic position of this genus within the CW group was ambiguous, possibly because of the limited numbers of operational taxonomic units (OTUs) analyzed (Naka-

zawa et al. 2004). Therefore, sequence data of *Asterococcus* are needed for genes that have been determined in many taxa in the CW group. Although Pröschold et al. (2001) studied the phylogeny of 95 strains of the CW group based on 18S ribosomal (r) RNA genes, *Asterococcus* sequences were not analyzed. Therefore we evaluated the phylogenetic position of the genus *Asterococcus* based on 18S rRNA sequences. Our results suggest that *Asterococcus* is separated from other members of the Tetrasporales within the CW group.

Material and Methods

Two strains of *Asterococcus* were selected as representatives of the genus since they have different colonial forms and belong to the two subgroups constituting the monophyletic *Asterococcus* group in the *rbcL* gene phylogeny (Nakazawa et al. 2004). They were *A. superbis* IAM C-299 with a spherical colony and *A. korschikoffii* Ettl ACOI 326 with a dendroid colony (Nakazawa et al. 2004) (Table 1).

The methods for DNA extraction and direct sequencing of polymerase chain reaction (PCR) products were the same as those of a previous study (Nakazawa et al. 2004), except for the primers used for PCR and se-

quencing of 18S rRNA genes (Table 2).

Ninety five OTUs in the CW group examined in this study were the same as those of Pröschold et al. (2001), and five additional CW OTUs are listed in Table 1. Seven OTUs of the directly opposed (DO) group (Fig. 1) were selected from Pröschold et al. (2001). The alignment of the 18S rRNA genes from the 95 CW and seven DO OTUs was extracted from that of Pröschold et al. (2001) and the five additional OTUs (Table 1) were realigned using Clustal X (Thompson et al. 1997).

The alignment (1642 bp; see Pröschold et al. 2001) was subjected to unweighted maximum parsimony (MP) analysis, including bootstrap analysis (Felsenstein 1985) with 100 replications of a full heuristic search based on the nearest-neighbor interchange (NNI) branch-swapping algorithm, using PAUP 4.0b10 (Swofford 2002). Following the guidelines for topology construction with distance methods (Nei and Kumar 2000), we selected Jukes-Cantor distances (Jukes and Cantor 1969) to construct neighbor-joining (NJ) trees. From the alignment used for the MP analysis, a Jukes-Cantor distance matrix was calculated (Jukes and Cantor 1969) using PAUP 4.0b10. With the NJ algorithm (Saitou and Nei 1987), a

Table 1. Additional OTUs of CW group (Pröschold et al. 2001) used for phylogenetic analysis using the 18S rRNA genes

Species	Strain designation	Origin of 18S sequence data	DDBJ/EMBL/GenBank accession number
<i>Asterococcus superbis</i>	IAM ¹ C-299	The present study	AB175836
<i>Asterococcus korschikoffii</i>	ACO ² 326	The present study	AB175837
<i>Characiochloris acuminata</i>	UTEX ³ 2095	Buchheim et al. (2002)	AF395435
<i>Hafniomonas montana</i>	NIES ⁴ -257	Nozaki et al. (2003)	AB101517
<i>Hafniomonas montana</i>	NIES-656	Nozaki et al. (2003)	AB101518

¹IAM Culture Collection at the University of Tokyo (Sugiyama et al. 1998).

²The Culture Collection of Algae at the Department of Botany, University of Coimbra (Santos and Mesquita 1986, Santos 1988, Santos et al. 1993).

³Culture Collection of Algae at the University of Texas at Austin (Starr and Zeikus 1993).

⁴Microbial Culture Collection at the National Institute for Environmental Studies (Watanabe et al. 2000).

Table 2. Primers used for amplifications and sequencing of the 18S rRNA genes

Designation	Positions ¹	Sequence (5'–3')
18S-FA	1–21	AACCTGGTTGATCCTGCCAGT
18S-RD	570–550 ²	GCTGGCACCAGACTTGCCCTC
18S-FG	557–577	AGTCTGGTGCCAGCAGCCGCG
18S-FE	1112–1132	GGGAGTATGGTCGCAAGGCTG
18S-RF	1202–1182 ²	CCCGTGTTGAGTCAAATTAAG
18S-RB	1799–1774 ²	TGATCCTTCTGCAGGTTACCTAC

¹Coordinate number from the *Chlorella vulgaris* Beijerinck 18S rRNA gene (Huss et al. 1990).

²Reverse primer.

phylogenetic tree was constructed, also using PAUP 4.0b10. The robustness of the resulting lineages was tested by bootstrap analysis with 1000 replications using PAUP 4.0b10. Using the same alignment data, a maximum likelihood (ML) analysis was carried out using PUZZLE in PAUP 4.0b10, with the HKY model (Hasegawa et al. 1985) to estimate quartet puzzling support values, which have the same practical meaning as bootstrap values (Strimmer and von Haeseler 1996) for internal branches of the phylogenetic tree with 1000 puzzling steps (comparable to the number of bootstrap replicates). In these phylogenetic analyses, the seven species in the DO group (Fig. 1) were designated the outgroup since the DO group is sister to the CW group containing volvocalean and biflagellate tetrasporalean algae (Booton et al. 1998).

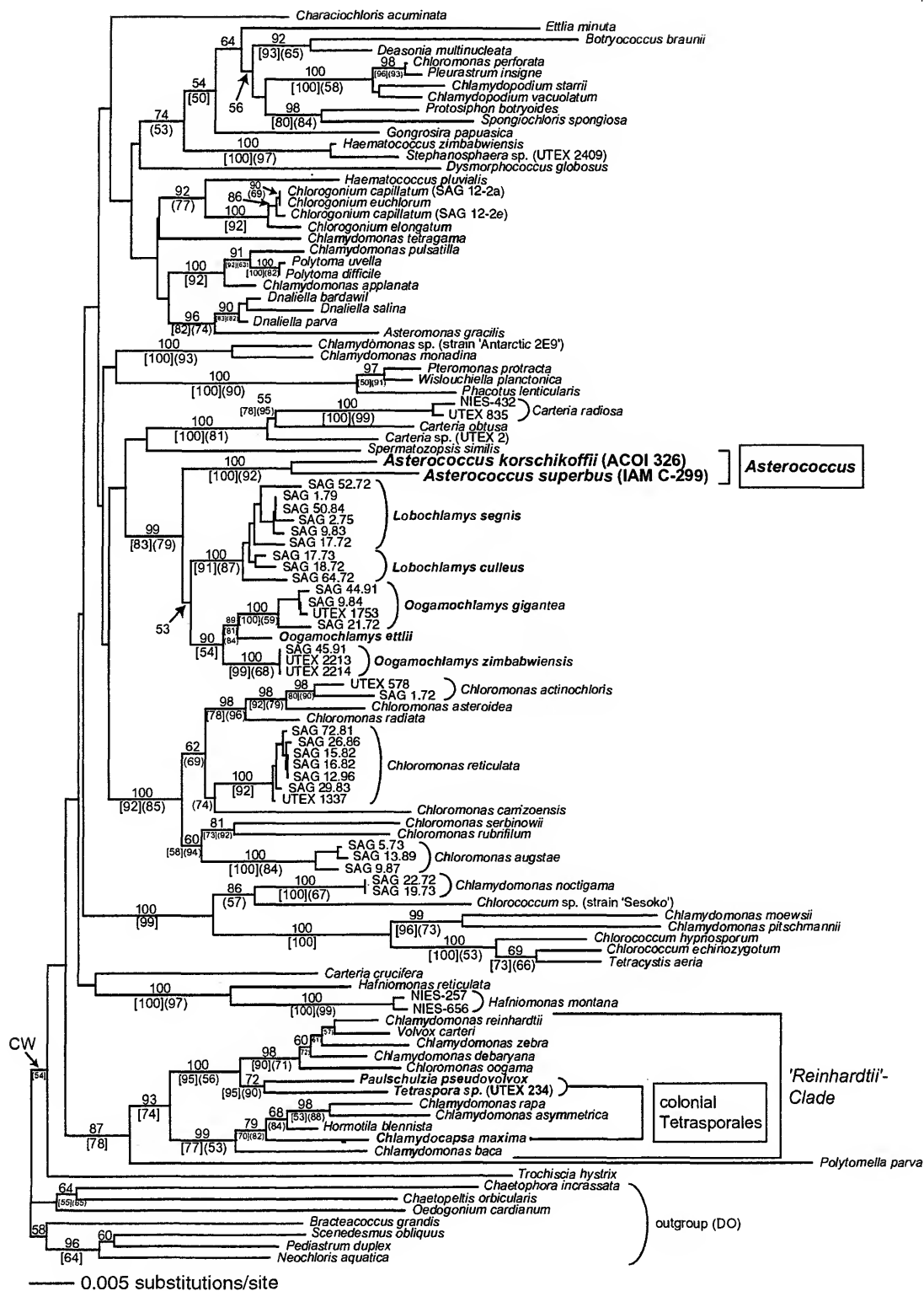
Results

The NJ tree of the 18S rRNA sequences is shown in Fig. 1, and branches with 50 % or greater bootstrap/QPS values in the NJ, MP and/or ML are shown. A robust monophyletic group consisting of the genus *Asterococcus* (*A. superbis* and *A. korschikoffii*) was resolved with high bootstrap/QPS values (92–100 %) using the NJ, MP, and ML methods. This genus was distant from the '*Reinhardtii*'-Clade (Pröschold et al. 2001), which included two separate

lineages of other tetrasporalean algae, one containing a palmellopsidacean species [*Chlamydocapsa maxima* (Mainx) Ettl & Gärtner] and the other composed of two colonial genera in the Tetrasporaceae (*Tetraspora* Link and *Paulschulzia* Skuja). By contrast, the genus *Asterococcus* constituted a robust monophyletic group with two unicellular volvocalean genera *Oogamochlamys* Pröschold & al. (2001) and *Lobochlamys* Pröschold & al. (2001) with high bootstrap/QPS values (79–99 %) with the NJ, MP, and ML methods. Within this group, the MP and ML analyses suggested that *Asterococcus* and *Oogamochlamys* formed a clade with 71–75 % bootstrap/QPS values (not shown), whereas the NJ analysis did not support this clade.

Discussion

Tetrasporales is traditionally characterized as having a gelatinous structure (gelatinous matrix and/or pseudoflagella) in the vegetative phase in which cells can be converted into reproductive cells without preceding cell divisions (e. g., Ettl and Gärtner 1988). However, the validity of this order has been doubted because no common characteristics clearly distinguish the Tetrasporales from the volvocalean and/or chlorococcalean green algae (e. g., van den Hoek et al. 1995). Based on the 18S rRNA gene phylogeny, Booton et al. (1998) demonstrated that the Tetra-



sporales is polyphyletic, composed of a biflagellate/CW group and a quadriflagellate/DO group within the Chlorophyceae. We clearly resolved three separate lineages of the tetrasporalean algae within the CW group (Fig. 1), and concluded that Tetrasporales is polyphyletic even within the CW group. Therefore, an immotile vegetative phase with gelatinous structures and the direct formation of the reproductive cells characterizing the Tetrasporales likely evolved in multiple phylogenetic positions in the CW group, and the taxonomic separation of Tetrasporales from Volvocales/Chlorococcales does not represent the natural phylogenetic relationships within the green algae.

Based on this study, *Asterococcus* and two biflagellate unicellular genera *Oogamochlamys* and *Lobochlamys* (Volvocales) form a robust monophyletic group within the CW group. However, no morphological features seem to characterize *Oogamochlamys/Lobochlamys* and *Asterococcus* at the light microscopic level (Pröschold et al. 2001, Nakazawa et al. 2004). Although the mucilage layer around the cell of *Lobochlamys* (Pröschold et al. 2001) seems to be homologous to the colonial gelatinous layers of *Asterococcus*, such a layer is also recognized in another unicellular genus, *Vitreochlamys*

in the CW group (e. g., Nakazawa et al. 2001). Patricia et al. (2000) and Nakazawa et al. (2004) reported details of the cell structure of *Asterococcus* using transmission electron microscopy. However, there are no morphological studies of *Oogamochlamys* and *Lobochlamys* based on ultrastructural observations. Further electron microscopic studies of these two genera may resolve ultrastructural features characterizing *Asterococcus* and *Oogamochlamys/Lobochlamys* and contribute to reconstruction of a natural taxonomic system of the CW green algae traditionally classified as members of the Tetrasporales.

We are grateful to Dr. T. Pröschold for providing the alignment of the 18S rRNA genes. This study was supported in part by a grant from the Institute for Fermentation (to HN).

References

- Booton G. C., Floyd G. L. and Fuerst P. A. 1998. Polyphyly of tetrasporalean green algae inferred from nuclear small-subunit ribosomal DNA. *J. Phycol.* **34**: 306–311.
- Buchheim M. A., Buchheim J. A., Carlson T. and Kugrens P. 2002. Phylogeny of *Lobochlamys* (Chlorophyceae) and allies: a study of 18S and 26S rDNA data. *J. Phycol.* **38**: 376–383.

Fig. 1. Distance tree based on aligned nucleotide sequences for 1642 bp in the 18S rRNA genes for two strains of *Asterococcus* and 98 other strains of CW group and seven DO algae. The tree was derived with the neighbor-joining method (Saitou and Nei 1987) based on the Jukes-Cantor distance (Jukes and Cantor 1969) indicated by the scale bar below the tree. Numbers above branches are the bootstrap values (50 % or more) based on 1000 replications. Numbers in brackets below the branches are bootstrap values resolved in the majority-rule (50 %) consensus tree of a bootstrap analysis based on 100 replications of most parsimonious analysis by PAUP 4.0b10. Numbers in parentheses below the branches are quartet puzzling support (QPS) values (50 % or more) of a maximum likelihood analysis with 1000 puzzling steps. Bootstrap/QPS values at distal branches within *Oogamochlamys*, *Lobochlamys* and *Chloromonas* are not shown. Designations of species names and the alignment of 18S rRNA genes are almost based on those of Pröschold et al. (2001). ACOI: The Culture Collection of Algae at the Department of Botany, University of Coimbra (Santos and Mesquita 1986, Santos 1988, Santos et al. 1993). IAM: IAM Culture Collection at the University of Tokyo (Sugiyama et al. 1998). NIES: Microbial Culture Collection at the National Institute for Environmental Studies (Watanabe et al. 2000). SAG: Sammlung von Algenkulturen at the University of Göttingen (Schlösser 1994). UTEX: Culture Collection of Algae at the University of Texas at Austin (Starr and Zeikus 1993).

- Ettl H. and Gärtner G. 1988. Chlorophyta II. Tetrasporales, Chlorococcales, Gloeodendrales. In: H. Ettl, J. Gerloff, H. Heynig and D. Mollenhauer (eds.), Süßwasserflora von Mitteleuropa vol. 10. 436 pp. G. Fischer, Stuttgart.
- Felsenstein J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* **38**: 16–24.
- Fott B. 1971. Algenkunde. 2. Aufl. 581 pp. VEG G. Fischer, Jena.
- Hasegawa M., Kishino H. and Yano T. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **22**: 160–174.
- Huss V. A. and Sogin M. L. 1990. Phylogenetic position of some *Chlorella* species within the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. *J. Mol. Evol.* **31**: 432–442.
- Jukes T. H. and Cantor C. R. 1969. Evolution of protein molecules. In: H. N. Munro (ed.), *Mammalian Protein Metabolism*. pp. 21–132. Academic Press, New York.
- Korshikov A. A. 1953. Protococcinae. In: Roll J. V. (ed.), *Viznacnik prsnovod. Vodoroslej T. 5*. 439 pp. Vid. akad. Nauk URSR, Kiev (in Ukrainian).
- Lemmermann E. 1915. Tetrasporales. In: Pascher A. (ed.), *Die Süßwasserflora Deutschlands, Österreichs und der Schweiz. H. 5*. pp. 21–51. G. Fischer, Jena.
- Nakazawa A., Krienitz L. and Nozaki H. 2001. Taxonomy of the unicellular green algal genus *Vitreochlamys* (Volvocales) based on comparative morphology of cultured material. *Eur. J. Phycol.* **36**: 113–128.
- , Yamada T. and Nozaki H. 2004 (in press). Taxonomic study of *Asterococcus* (Chlorophyceae) based on comparative morphology and *rbcL* gene sequences. *Phycologia*.
- Nei M. and Kumar S. 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
- Nozaki H., Misumi O. and Kuroiwa T. 2003. Phylogeny of the quadriflagellate Volvocales (Chlorophyceae) based on chloroplast multigene sequences. *Mol. Phylogenet. Evol.* **29**: 58–66.
- Patricia I. L., María C. R. and Eduardo J. C. 2000. Fine structure of biflagellate zoospores of *Asterococcus superbus* (Tetrasporales, Chlorophyceae), including the absolute configuration of the flagellar apparatus. *J. Phycol.* **36**: 714–722.
- Pröschold T., Marin B., Schlösser U. G. and Melkonian M. 2001. Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). 1. Emendation of *Chlamydomonas* Ehrenberg and *Chloromonas* Gobi, and description of *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. *Protist* **152**: 265–300.
- Saitou N. and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
- Santos M. F. 1988. ACOI—The culture collection of algae of the Department of Botany—University of Coimbra—Supplement—I. *Bol. Soc. Brot., sér. 2*, **61**: 267–277.
- and Mesquita J. F. 1986. The culture collection of algae of the Department of Botany—University of Coimbra. *Bol. Soc. Brot., sér. 2*, **59**: 353–373.
- , Lourenço O. and Carvalho G. 1993. ACOI—The culture collection of algae of the Department of Botany—University of Coimbra—Supplement—II. *Bol. Soc. Brot., sér. 2*, **66**: 173–200.
- Scherffel A. 1908. *Asterococcus* n. g. *superbus* (Cienk.) Scherffel und dessen angebliche Beziehungen zu *Eremosphaera*. *Ber. Deutsch. Bot. Ges.* **26a**: 762–771.
- Schlösser U. G. 1994. SAG-Sammlung von Algenkulturen at the University of Göttingen. Catalogue of strains 1994. *Bot. Acta* **107**: 111–186.
- Smith G. M. 1950. *The Fresh-water Algae of the United States*. 2nd ed. 719 pp. McGraw-Hill, New York.
- Starr R. C. and Zeikus J. A. 1993. UTEX—the Culture Collection of Algae at the University of Texas at Austin. *J. Phycol.* **29**: 1–106 (Suppl.).
- Strimmer K. and von Haeseler A. 1996. Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* **13**: 964–969.
- Sugiyama J., Yokota A., Unami E., Onodera Y., Nishida H., Miura Y., Isobe M., Kasahara Y. and Sakamoto K. 1998. IAM. catalogue of strains, 2nd ed. The Microbiology Research Foundation, Tokyo.
- Swofford D. L. 2002. PAUP* 4.0: phylogenetic analysis using parsimony, version 4.0b10. Computer program distributed by Sinauer Associates, Inc., Fitchburg.
- van den Hoek C., Mann D. G. and Jahns H. M. 1995. *Algae: an introduction to phycology*. 627 pp. Cambridge Univ. Press, Cambridge.
- Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. and Higgins D. G. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **25**: 4876–4882.
- Tiffany L. H. 1934. *The Plankton Algae of the West End of Lake Erie*. The Ohio State University, the Franz Theodore Stone Laboratory, contr. No. 6.

112 pp. The Ohio State University Press, Columbus.
Watanabe M. M., Kawachi M., Hiroki M. and Kasai F.

2000. NIES-collection. List of Strains. Algae and Protozoa, 6th ed. 159 pp. National Institute for Environmental Studies, Tsukuba.

中沢 敦, 野崎久義: 18S rRNA 遺伝子配列を用いたヨツメモ目 *Asterococcus* 属 (緑藻綱) の分子系統解析

Asterococcus Scherffel は放射状の葉緑体と細胞群体を包む膨潤した寒天状基質層により特徴付けられる不動性微細藻であり, 緑藻綱, ヨツメモ目, パルメロプシス科に分類されている. 筆者らは本属の緑藻綱の CW グループ (鞭毛基部の配置が時計回り型のボルボックス目, クロロコクム目の一部, 2 鞭毛性ヨツメモ目を含む一群) 内における系統的位

伝子1642塩基対を用いた系統解析を行った. その結果, *Asterococcus* は遊泳性の単細胞性ボルボックス目の *Lobochlamys* 及び *Oogamochlamys* と高いブートストラップ確率で単系統をなし, *Tetraspora* を含むヨツメモ目のクレード及び, パルメロプシス科に属する *Chlamydocapsa maxima* (Mainx) Ettl & Gärtner と分離することが示された.

(東京大学大学院理学系研究科生物科学専攻)